

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/39, 39/02, 9/16, 9/51, A61P 31/04	A2	(11) International Publication Number: WO 00/56361 (43) International Publication Date: 28 September 2000 (28.09.00)
(21) International Application Number: PCT/GB00/01104 (22) International Filing Date: 23 March 2000 (23.03.00) (30) Priority Data: 9906694.6 24 March 1999 (24.03.99) GB 9906696.1 24 March 1999 (24.03.99) GB (71) Applicant (for all designated States except US): THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation and Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): ALPAR, Hazire, Oya [GB/GB]; Aston University, Aston Triangle, Birmingham B4 7ET (GB). SOMAVARAPU, Satyanarayana [IN/GB]; Aston University, Aston Triangle, Birmingham B4 7ET (GB). WILLIAMSON, Ethel, Diane [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). BAILLIE, Leslie, William, James [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). (74) Agent: BOWDERY, A., O.; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).	(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: VACCINE COMPOSITION (57) Abstract A pharmaceutical composition comprising: (i) a biologically active agent; (ii) an adjuvant chemical which increases the effect of the biologically active agent, said chemical selected from one or more of: A) a polyamino acid, B) a vitamin or vitamin derivative, C) cationic pluronics, D) a clathrate, E) a complexing agent, F) cetrimides, G) an S-layer protein, or H) methyl-glucamine; (iii) a pharmaceutically acceptable carrier or diluent, provided that when the chemical (ii) above is selected from D) or E), the biologically active agent is an agent which is capable of generating a protective immune response in an animal to which it is administered. The composition, which may be in the form of a solution or particles such as microspheres or liposomes, is particularly useful for mucosal administration of vaccines especially be the intra-nasal route or by parenteral routes.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Vaccine Composition

The present invention relates to compositions which are particularly useful for delivering medicaments, and in particular vaccines. Compositions are suitable for parenteral administration or non-parenteral administration. This includes compositions for administration to mucosal surfaces, for example intranasal formulations, or for topical administration to the skin. The invention further comprises methods of treating individuals using the composition, methods of preparing the composition and components for use in the compositions.

A prime objective in the field of vaccination is the development of a non-parenteral immunisation regimen which facilitate induction of comparable levels of systemic immunity to that elicited by conventional sub-cutaneous and intra-muscular injections.

The nasopharyngeal passages and pulmonary regions of the respiratory tract represent potential targets for the systemic delivery of peptidergic drugs and vaccines. The relative ease with which therapeutic agents can be inhaled, or introduced into the nose, make these modes of immunisation attractive in terms of probable patient compliance. Furthermore, respiratory mucosae offer certain morphological, physiological and immunological advantages over other non-parenteral sites in terms of immunisation, particularly against pathogenic entities which affect or utilise mucosal surfaces as portals of entry. This is because effective vaccination against these pathogens normally requires mucosae to be adequately protected with locally produced antibodies of the secretory IgA (sIgA) isotype. Whilst mucosal surfaces are usually poorly protected with IgA following parenteral administration of vaccines, it is now apparent that successful delivery of antigenic material to immunoresponsive elements in mucosa-associated lymphoid tissue (MALT) can result in vigorous stimulation of the mucosal arm of

the immune system. By means of the common mucosal immune system (CMIS) it is feasible that several anatomically disparate mucosal surfaces could be protected through mucosal administration of a vaccine at a single site. Mucosal vaccination offers the added advantage that some degree of systemic immunity can be induced in concert with local responses due to translocation of antigenic material from sub-epithelial compartments to systemic immunoresponsive tissues.

Despite the logistical and immunological factors which favour non-parenteral immunisation, simple mucosal application of antigenic proteins, for example in the gastrointestinal or respiratory tracts, is usually ineffectual in terms of vaccination. Enzymatic or chemical destruction, combined with poor absorption into sub-epithelial compartments dictate that mucosally administered vaccines usually require some form of adjuvant or delivery vehicle. One approach is to encapsulate antigenic material within microparticulate polymeric carriers, such as poly-DL-lactide (PLA) microspheres (Vaccine 1994, 12, 5-11). Such procedures serve to protect labile vaccines from luminal degradation and enhance absorption into mucosal and systemic compartments (J.H. Eldridge et al., Seminars in Hematology, (1993), 30, 16-25). There is good evidence that microencapsulation may also adjuvantise by converting soluble antigenic molecules into particulate species, thus promoting vaccine uptake into antigen presenting cells (APC) (Y. Tabata et al., Adv. Polym. Sci. (1990), 94, 107-141, L. Vidard et al., J. Immunol. (1996), 156, 2809-2818, N. Van Rooijen, Immunol. Today (1990) 11, 436-439) or microfold cells (M-cells) in lymphoid follicles (R.I. Walker et al., Vaccine, 12, 387, 1994, D.T. O'Hagan et al., Vaccine, 1989, 7, 421-424, P.G. Jenkins et al., J. Drug Targeting, 1995, 3, 79-81). Nasal delivery of microsphere formulation of vaccine has also been reported (A.J. Almeida et al., J. Pharm & Pharmacology, 25, 198-203 1993, H.O. Alpar et al., J. Drug Targeting 2/2, 147-149, 1994, A.J. Almeida et al., J. Drug Targeting 3(b), 255-467 1996).

Although until recently comparatively under-investigated, the intra-nasal (i.n.) route is an attractive one for the mucosal delivery of vaccinal entities. The nasal epithelium is
5 accessible and is less exclusive to high molecular weight molecules.

The thickness of the mucus blanket covering respiratory epithelium is relatively thin compared to that of other mucosae,
10 for example the gut where it is in the region of 500 times thicker. Substantially reduced concentrations of proteolytic enzymes and extremes of pH exist in the respiratory tract compared with the gastrointestinal tract.

15 Furthermore, it is now delineated that nasal associated lymphoids tissues (NALT) have a lymphoepithelium which, like that in the intestinal mucosa, contain M-cells for selective antigen uptake (P. Brandenburg, Immunology of the Lung and Upper Respiratory Tract, (ed. Bienenstock J.) McGraw-Hill, New York,
20 1984, 28-95). Hence NALT plays an analogous role to other MALT, such as the gut associated lymphoid tissues (GALT), in terms of antigen surveillance and induction of mucosal and systemic immunological responses.

25 The applicants have found that a particular range of chemicals, when included in formulations, can increase or enhance the effect of biologically active agents and in particular vaccines. A particular effect noted appears to be an immunostimulant or adjuvant effect. They are noted particularly when the
30 compositions are administered to mucosal surfaces, but are also observed when compositions are applied parenterally, for example intramuscularly (i.m. administration). Topical formulations for administration to skin surfaces may also benefit from the inclusion of these chemicals.

As used herein, the term "immunostimulant" refers to an adjuvant which stimulates the immune system of a host animal to which it is administered and thereby increases the protective effect produced by a protective antigen administered to that animal, as compared to the effect which would be produced by administration of the protective antigen alone.

According to the present invention there is provided a pharmaceutical composition comprising

- 10 (i) a biologically active agent;
 - (ii) an adjuvant chemical which increases the effect of the biologically active agent, said chemical selected from one or more of:
 - 15 A) a polyamino acid,
 - B) a vitamin or vitamin derivative,
 - C) cationic pluronics,
 - D) a clathrate,
 - E) a complexing agent,
 - F) cetrimides;
 - 20 G) an S-layer protein; or
 - H) Methyl-glucamine; and
 - (iii) a pharmaceutically acceptable carrier or diluent;
- provided that when the chemical (ii) above is selected from D) or E), the biologically active agent (i) is an agent which is capable of generating a protective immune response in an animal to which it is administered.

Particular examples of suitable chemicals are those of groups, A, B, C, D, E, F and H defined above. A further particular group of chemicals are those of group G above.

As used herein, the expression "cationic pluronics" includes both pluronics which include cations, as well as those which have been treated such that they are bound to cationic moieties (cationised pluronics).

Suitable biological agents (i) include drugs and therapeutic molecules such as vaccines, antivirals, antibiotics, antifungals, antiparasitics as well as oligonucleotides used in therapies and vaccines.

5

However in a preferred embodiment, the biologically active agent is an agent that is capable of generating an immune response in an animal to which it is administered and most preferably a protective immune response. Thus the compositions are suitably used as vaccines including those which rely on oligonucleotides or other nucleic acid sequences. In this case, the immunostimulant properties of the compounds A-H are used.

Suitably the said adjuvant chemical is soluble in water. Suitably the composition is suitable for non-parenteral administration for example to mucosal surfaces or for topical application to the skin. Particularly preferred compositions are suitable for administration to mucosal surfaces.

Alternatively, the composition is suitable for parenteral administration for example by intramuscular (i.m.) administration.

Administration to mucosal surfaces may be effected by oral application, by pulmonary application, for example by intra-tracheal administration, or particularly by intra-nasal application. In particular, the compositions of the invention are administered by the intra-nasal route.

Examples of adjuvant chemicals in category (A) above include polyamino acids such poly-ornithine, for example of molecular weight from 5 to 150kDa.

Particular examples of adjuvant chemicals in category (B) above are vitamins or vitamin derivatives such as vitamin E or

derivatives for example vitamin E TPGS (d-alpha tocopheryl polyethylene glycol 1000 succinate).

Particular cationic pluronics in category (C) above, are block
5 copolymers or surfactants which are positively charged, in particular with NH_2^+ groups. These are available commercially for example from ICI Ltd (UK) sold under the trade names P101 and P121. These may be used alone, but may preferably be used in combination with other adjuvants.

10

Examples of clathrates in category (D) above include in particular cyclodextrins and their derivatives such as dimethyl β cyclodextrin.

15 Suitable complexing agents in category (E) above are bile salts, in particular those which form complexes with fatty acids such as deoxycholic acid.

Examples of cetrimides in category (F) are quaternary ammonium
20 compounds used as preservatives.

Such chemicals may further enhance the mucosally produced effect of the biologically active agent by acting as absorption enhancers and/or bioadhesive compounds and/or solubilisers.

25

Preferably the chemicals include at least one of the group selected from (A), (B), (C) or (D). When the chemical comprises a vitamin or derivative of (B) above, it is suitably present in concentrations in excess of 0.2%w/v, preferably in
30 excess of 2%w/v.

Carriers or diluents used as (iii) above may vary depending upon the particular nature of the biologically active agent (i) and the further chemical (ii). They may comprise pharmaceutically
35 acceptable solvents such as water in which the biologically active agent (i) and the further chemical (ii) are dissolved.

This type of formulation is particularly suitable when (i) is also water-soluble.

Compositions in the form of solutions of this type suitably
5 contain from 0.1 to 30% w/v and preferably from 1 to 20% w/v of
component (ii) above, depending upon the solubility of component
(ii).

For many applications however, it has been found preferable that
10 components (i) and (ii) are microencapsulated in a polymeric
material and thus the carrier (iii) is a particulate carrier
such as a microparticle, nanocapsule or liposome.

Thus in a particular embodiment, the invention provides a
15 pharmaceutical composition, which composition comprises
particles comprising
(i) biologically active agent;
(ii) an adjuvant chemical which increase the biological effect
of the composition, said chemical being soluble in water and
20 being selected from one or more of:
A) a polyamino acid; or
B) a vitamin or vitamin derivative; and
C) cationic pluronic
D) a clathrate,
25 E) a complexing agent,
(iii) a material capable of forming a particle.

Particular examples will include a compound of (A), (B), (D) or
(E). Cationic pluronics of (C) may be included either within
30 the particles, or as a component of the dosing mixture or both.

Compositions of this type, are, as mentioned above, particularly
suitable for administration to a mucosal surface, although they
may be of a suitable size to allow parenteral administration.

Particularly suitable particles are liposomes and microspheres. Liposome forming chemicals for use as (iii) above are well known in the art and include lipids with a hydrophilic end region and a hydrophobic region and the opposite end of the molecule.

5 Microspheres or microparticles (sometimes called microcapsules) will generally be prepared using polymeric materials as is known in the art.

10 Suitably, the adjuvant chemical which increases the biological effect of the composition in this case is a polymeric material which is different to the polymeric material, where present, of item (iii) above.

15 The polymeric material (iii) above used in the compositions of the invention may comprise one or more polymers, for example having molecular weights of from 2kDa or more. In particular, the polymeric material (iii) is a high molecular weight polymer, for example of molecular weight in excess of 94kDa, for example of 100kDa or more.

20 The use of high molecular weight polymers in the encapsulation of a tetanus vaccine for intramuscular administration has been described (Vaccine 1994, 12, 4, 299-306). A formulation of microencapsulated ricin toxoid vaccine which is applied
25 intranasally has also been described (Vaccine 1994, 14, 11 1031). However, in that case, high molecular weight polymer microparticles (94kDa) were less effective than those prepared from a copolymer of lower molecular weight (72kDa).

30 A particularly suitable polymeric material for use in the compositions of the invention comprises poly-(L-lactide) or PLA but other polymeric materials such as poly(lactic/glycolic acid) PLGA, polycyanacrylates, polyanhydrides or polycaprolactones as are known in the art may be employed.

35

Suitably the component (ii) is present in the composition in an amount of from 0.1% to 10%w/w.

The compositions of the invention may optionally further
5 comprise agents which stabilise emulsions such as polyvinylalcohol or methyl cellulose.

Other conventional reagents may be added. These include other known composition components such as colouring agents and
10 preservatives and in particular cetrimide. These are suitably present in amounts of from 0.1 to 0.7%w/v.

In a particular embodiment, the microspheres or liposomes used in the compositions may comprise S-layer proteins. These may be
15 present as the sole immunostimulant compound, or they may be used in combination with other immunostimulants such as those defined in A)-F) and (H) above. The S-layer proteins may be present in the microspheres or liposomes themselves. In a preferred embodiment however, they are provided as a coating on
20 the surface of the microspheres or liposomes. Particularly preferred S-layer proteins are derived from a bacterium against which the biologically active agent produces a protective immune response. It has been shown (Sleyr et al., Crystalline bacterial cell surface proteins. Biotechnology Intelligence
25 Unit, 1996, R.G. Landes Company and Academic Press Inc.) that the stability of liposomes can be increased by such coatings. S-layer proteins are found on the surface of most bacteria and form a regular two dimensional array known as an S-layer. Isolated S-layer proteins are able to form entropy driven
30 monomolecular arrays in suspension, and on the surface of structures such as liposomes.

S-layer proteins have not heretofore, been applied to microspheres however. Consequently, microspheres containing
35 such proteins, in particular in the form of a coating on the surface of the microsphere, form a further aspect of the

invention. These microspheres are preferably utilised as vaccines in that they carry one or more immunogenic agents which produce a protective immune response when administered to an animal. These agents may be within or distributed throughout the
5 microsphere and may additionally be complexed to the S-layer protein coating.

Preferably, the S-layer protein is derived from the bacterial pathogen against which protection is sought. For example,
10 microspheres employed as in vaccines against *B.anthraxis* may be coated with *B. anthracis* S-layer protein.

Compositions of the invention are particularly suitable for mucosal, especially intranasal application. They may comprise
15 simple solutions of the components as described above, or microparticles per se which are optionally preserved, for example by lyophilisation, or the microparticles may themselves be combined with a pharmaceutically acceptable carrier or excipient. Examples of suitable carriers include solid or
20 liquid carriers as is understood in the art.

Microparticles used in the compositions of the invention will suitably be of an average size of from 0.1µm to 10µm in diameter.
25

These compositions may be used to deliver a range of biologically active agents including drugs and pharmaceutical chemicals as well as hormones such as insulin.

30 These compositions have been found to be particularly effective in the administration of biologically active agent which is capable of generating a protective immune response in an animal, particularly a mammal, to which it is administered. Examples of such agents include antigenic polypeptides as well as nucleic
35 acid sequences which may encode these polypeptides and which are known as "DNA" vaccines. Both the level and the longevity of

the immune response is increased when these formulations are employed.

As used herein the expression "polypeptide" encompasses proteins
5 or epitopic fragments thereof.

Suitable polypeptides are sub-unit vaccines and others, such as diphtheria toxoid, tetanus toxoid and *Bacillus anthracis* protective antigen (PA).

10

In a preferred embodiment, the composition of the invention comprises a biologically active agent which is capable of generating a protective immune response against *Yersinia pestis*. The agent is suitably a sub-unit vaccine, for example as
15 described in WO 96/28551. The vaccine described and claimed there comprises a combination of the V antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, and the F1 antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these.

20

As used herein, the term "fragment" refers to a portion of the basic sequence which includes at least one antigenic determinant. These may be deletion mutants. One or more epitopic region of the sequence may be joined together.

25

The expression "variant" refers to sequences of nucleic acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may
30 be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible
35 without altering the biological activity of the polypeptide. Suitably variants will be at least 60% homologous, preferably at

least 75% homologous, and more preferably at least 90% homologous to the base sequence. Homology in this instance can be judged for example using the algorithm of Lipman-Pearson, with Ktuple:2, gap penalty:4, Gap Length Penalty:12, standard
5 PAM scoring matrix (Lipman, D.J. and Pearson, W.R., Rapid and Sensitive Protein Similarity Searches, *Science*, 1985, vol. 227, 1435-1441).

Preferably, vaccine compositions will further comprise a further
10 known adjuvant in order to enhance the immune response to the biologically active material administered. Suitable adjuvants include pharmaceutically acceptable adjuvants such as Freund's incomplete adjuvant, alhydrogel, aluminium compounds and, preferably adjuvants which are known to up-regulate mucosal
15 responses such as CTB, the non-toxic pentameric B subunit of cholera toxin (CT), or mutant heat-labile toxin (mLT) of *E.coli*.

A further potential immunostimulant compound which may be included in the compositions are polycationic carbohydrates such
20 as those described and claimed in copending International Patent application of the applicants of even date, derived from British Patent Application Nos. 9906694.6 and 9906696.1. Particular examples of polycationic carbohydrate which act as immunostimulants include a chitin derivative, cationic
25 polypeptide, cationic polyamino acid, a quaternary ammonium compound or a mixture thereof. Especially preferred are chitin derivatives such as chitosan, or water-soluble chitin derivatives such as alkylated chitosans or salts thereof. Particular examples are trimethylchitosans such as those
30 described by A.F. Kotze et al. *Pharm Res.* (1997) 14: 1197-1202.

A particular aspect of the invention comprises a method of producing a pharmaceutical composition, which method comprises encapsulating a biologically active agent in a particle
35 comprising a first material which is capable of forming a particle, in the presence of an adjuvant chemical which

increases the effect of the biologically active agent when administered to a mucosal surface, said chemical being soluble in water and being selected from one or more of:

- 5 A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- F) cetrimides; or
- 10 H) Methyl-glucamine.

In particular the said chemical is selected from one or more of

- A) a polyamino acid;
- B) a vitamin or vitamin derivative,
- 15 D) a clathrate,
- E) a complexing agent.

In an alternative group, the said chemical is selected from one or more of

- 20 A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,

- 25 Preferred examples of particle forming materials and adjuvant (effect enhancing) chemicals are as set out above.

Methods of forming liposomes are well known in the art. They include dispersion of dehydrated phospholipid films into an aqueous medium, emulsion techniques and lyophilisation methods as are well known in the art.

30

The adjuvant chemical may be incorporated within the microcapsule, or at the surface, of preferably is distributed throughout the microcapsule including the surface.

35

Microencapsulated compositions of the invention are suitably prepared using a double emulsion solvent evaporation method. Briefly, the biologically active agent, suitably in a lyophilised state, is suspended in an aqueous solution of the polymer such as polyvinyl alcohol (PVA) and the adjuvant chemical. A solution of further polymer, in particular high molecular weight polymer in an organic solvent such as dichloromethane, is added with vigorous mixing. The resultant emulsion is then dropped into a secondary aqueous phase, also containing polymer (PVA or the like) and optionally also the adjuvant material with vigorous stirring. After addition, the organic solvent is allowed to evaporate off and the resultant microspheres separated.

The compositions of the invention will suitably comprise an appropriate dosage unit of the active agent. This will vary depending upon the nature of the active agent being employed, the nature of the patient, the condition being treated and other clinical factors. In general however, the composition of the invention will comprise approximately 2 to 10 wt% of active ingredient.

In microcapsule formulations, the amount of high molecular weight first polymer in the composition will be of the order of 70 to 99wt% of the composition, and suitably from 90 to 99wt% of the polymer components will be the first polymer.

The amount of adjuvant chemical present in the compositions will be sufficient to produce the required effect. This will vary depending upon the nature of the chemical but will generally be of the order of 0.1 to 10 wt % of the composition.

In use, a reasonable dosage for nasal administration would be from about 0.05g to 0.2g.

Preferred compositions of the inventions are vaccine compositions. Thus, in a further aspect, the invention provides a method of protecting a mammal against infection, which method comprises administration of a vaccine composition as described
5 above in particular to a mucosal surface, such as a nasal surface, of a mammal.

The applicants have demonstrated that it is possible to protect experimental animals from inhalation challenge with various
10 pathogens including diptheria, tetanus and *Y. pestis* through i.n. administration of a combined sub-unit vaccine. The adjuvantisation of these sub-units is advantageous in increasing the immune response as is microencapsulation of the sub-units in accordance with the invention. The high molecular weight
15 polymer utilised in the compositions of the invention appears to be particularly well suited to intra-nasal delivery.

In a further aspect, the invention provides the use of an adjuvant chemical as defined above as an immunostimulant in the
20 production of a vaccine for use in prophylactic or therapeutic treatment.

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:
25

Figures 1 illustrates the specific serum antibody responses following a single nasal application of 1µg V and 5µg F1 antigens of *Yersinia pestis* in compositions according to the invention:
30

Figures 2-4 illustrates the immune response to nasally delivered tetanus toxoid (TT) using compositions according to the invention where BS is glycodeoxycholic acid, CYC is dimethyl β cyclodextrin, and VET is Vitamin E TPGS and PO is polyornithine;
35

Figures 5-6 illustrate the immune response to nasally delivered Diptheria toxoid (DT) using compositions according to the invention where BS is glycodeoxycholic acid, CYC is dimethyl β cyclodextrin, and VET is Vitamin E TPGS;

5

Figure 7 shows the results of photon correlation spectroscopy (PCS) on particles produced for use in the invention, showing that the number mean diameter (d_n) and the volume mean diameter (d_v) were both around 150nm; and

10

Figure 8 illustrates the immune response to Diptheria toxoid (DT) in various formulations applied by i.m. routes, where P101 is a pluronic 101 and P121 is a block co-polymer available from ICI Ltd, UK.

15

Example 1

The effect of adjuvant chemicals.

Twelve groups of five (n=5) BALB/c mice were intranasally immunised with admixed F1 (5 μ g) and V (1 μ g). The five treatment groups received the subunits in conjunction with either:

20

- 1 Antigens free in 138kDa polyornithine (0.2-1%w/v conc.)
- 2 Microspheres including 138kDa polyornithine (0.2-1%w/v conc.)
- 3 Free in 30-70KDa polyornithine (0.2%w/v)
- 4 Free in 5-15kDa polyornithine (0.2%w/v)
- 5 Free in β -cyclodextrins (2.5%w/v)
- 6 Microspheres in β -cyclodextrins (2.5%w/v)
- 7 Free in deoxycholic acid (0.25%w/v)
- 8 Free in Vitamin E TPGS (2.5%w/w)
- 9 Free in Vitamin E TPGS (0.2% w/v)
- 10 Free in chitosan HCL (0.2%w/v)
- 11 Free in phosphate buffered saline (PBS)
- 12 Microspheres in PBS

A further group of animals acted as a control.

Mice were lightly anaesthetised with an inhaled gaseous mixture of 3% (v/v) halothane (RMB Animal Health Ltd., UK) in oxygen (300cm³ min⁻¹) and nitrous oxide (100cm³ min⁻¹) for i.n. dosing procedures. Each mouse received a 15µl volume of liquid administered with a micropipette. Tail vein blood samples were taken on day 14, and serum was analysed for the presence of anti-V and anti-F1 IgG antibodies using an indirect ELISA protocol (Eyles, J. E. et al. Vaccine (1998) 16:698-707).

The results are shown in Figure 1. This clearly shows that other compounds, in particular, poly-L-ornithine either free or in microspheres, β-cyclodextrins, deoxycholic acid and Vitamin E TPGS (the latter being present in amounts of 2.5% w/v) produced enhanced results.

This study has identified that absorption enhancers, with potential applications for increasing the bioavailability of non-parenterally administered peptidergic drugs, can also act to improve humoral immunity to mucosally applied subunit vaccines.

Example 2

Immune responses to nasally delivered tetanus toxoid (TT)

Further tests were carried out using the methodology of Example 1 but replacing the *Yersinia pestis* antigens with tetanus toxoid. Mice were dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid. The toxoids were in solution in combination with a variety of adjuvant chemicals in various concentrations. The results are shown in Figures 2-4.

With these enhancers or immunostimulants, titres for primary responses were improved approximately 100 times and secondary responses between 1000 to 20000 times compared to free antigen.

Example 3

Immune responses to nasally delivered diphtheria toxoid (DT)

Example 2 was repeated on selected members of the enhancers using diphtheria toxoid in place of tetanus toxoid. The results are shown in Figures 5 and 6. Again, similar levels of enhancement are noted.

5

Example 4

Immune responses to intramuscularly delivered diphtheria toxoid (DT)

Eight formulations of pluronic/chitosan nanoparticles were prepared, by a simple sonication method. Deacylated high molecular weight chitosan was obtained from Fluka. Three process variables were : type of Pluronic used (P101, P121 obtained from ICI Limited, UK), volume of pluronic added (75µl or 200µl per 2ml water), and addition or omission of chitosan. Briefly, to 2ml of double-distilled water, a small volume (75µl or 200µl) of the appropriate pluronic liquid (P101 or P121) was added. Mixtures were vortexed for one minute and sonicated for a further one minute. For coating of the pluronic particles, 100µl of a solution of 0.1% w/v high molecular weight chitosan in 2% w/v glacial acetic acid was added to 100µl of each formulation. Finally, the diphtheria toxoid (DT) was adsorbed to the 200µl of coated and non-coated particles by the addition of 12µl of a solution of DT in water (4450lf units per ml). This preparation is a colloidal dispersion in water with a mean particle diameter of generally between 100-600nm. An example of a typical photon correlation spectroscopy (PCS) printout is shown in Figure 7.

Following characterisation of the prepared particulate formulations, groups of four or five female Balb/c mice were given a single dose of 50µl intramuscularly. The total equivocal dose for each animal was 5Lf units of DT. The final concentration of pluronics in the dosing medium was 5% (v/v) and chitosan 0.05% (w/v). Animals were bled periodically and ELISAs

carried out to determine serum levels of anti-DT specific IgG titres.

Mean serum anti-DT IgG titres are shown herewith in Figure 8.

- 5 Surprisingly it was found that chitosan-pluronic composition produced much higher immune response compared to pluronics or with free antigen alone.

Example 5

- 10 S-Layer protein extraction and purification from *Bacillus anthracis* strains RBA91 and SM91 and microspheres containing them

- Bacillus anthracis* produces two S-layer (surface layer) proteins: EA1 (Extractable Antigen) and Sap (Surface Array Protein) which form ordered paracrystalline arrays exterior to the cell-wall (Etienne-Toumelin et al. 1995: Journal of Bacteriology, 177(3): 614-620; Farchaus et al. 1995, Journal of Bacteriology 177:2481-2489 and Mesnage et al. 1997, Molecular Microbiology, 23(6):1147-1155). The proteins may be isolated using the method of Etienne-Toumelin et al. 1995 supra.

- In this method SPY medium (Etienne Toumelin et al. 1995 supra.) agar plates, supplemented with spectinomycin, are inoculated with the *B. anthracis* mutant strains RBA91 and SM91 (Mesnage et al, 1997 supra.). The plates are incubated overnight at 37°C.

- Late the next day, some of the biomass from each plate is re-suspended in sterile saline to an OD₅₄₀ of approximately 1.3 in each case. Prewarmed SPY broth (500ml in 1000ml flasks), supplemented with spectinomycin, was prepared fresh for each of the two mutants, saline suspension (400µl) was used to inoculate each flask. The cultures were then incubated with shaking at 37°C, at approximately 200rpm for 16-18 hours.

- 35 OD₅₄₀ readings were then taken from each culture and those with results of 1.5 or more selected. The culture broths were

decanted into sterile Sorvall/Beckamann centrifuge pots and centrifuged at 10000rpm for 30 minutes at 4°C. The supernatant was discarded and the pellets re-suspended in 5M guanidine hydrochloride at pH 8.0 (25ml) in sterile 50ml Falcon tubes.
5 The re-suspended pellet mixtures were incubated for 2 hours at room temperature in a shaking incubator at approx. 150 rpm.

The extracts were then decanted into sterile 40ml Sorvall/Beckmann centrifuge tubes and centrifuges at 6000 rpm,
10 for 10 minutes at 4°C. The supernatants were decanted into sterile 30ml Universals. A set of 15ml Slide-a-Lyzer™ dialysis cassettes (Pierce, USA) are prepared and 15ml volumes of the supernatants are loaded by syringe needle into each cassette. Each cassette is placed in 1litre of 26mM Tris-HCl at pH 5.0 in
15 a glass beaker. The samples were dialysed overnight at 4°C in a refrigerator.

The S-layer proteins obtained in this way were then adsorbed onto microspheres which contained *B. anthracis* protective
20 antigen (PA) as the biologically active agent.

Claims

1. A pharmaceutical composition comprising
- (i) a biologically active agent;
 - 5 (ii) an adjuvant chemical which increases the effect of the biologically active agent, said chemical selected from one or more of:
 - A) a polyamino acid,
 - B) a vitamin or vitamin derivative,
 - 10 C) cationic pluronics,
 - D) a clathrate,
 - E) a complexing agent,
 - F) cetrinides;
 - G) an S-layer protein; or
 - 15 H) Methyl-glucamine
 - (iii) a pharmaceutically acceptable carrier or diluent, provided that when the chemical (ii) above is selected from D) or E), the biologically active agent is an agent which is capable of generating a protective immune response in an animal
 - 20 to which it is administered.
2. A composition according to claim 1 wherein biologically active agent is an agent that is capable of generating a protective immune response in an animal to which it is
- 25 administered.
3. A composition according to claim 1 or claim 2 wherein the said adjuvant chemical can act as an immunostimulant.
- 30 4. A composition according to any one of the preceding claims wherein the said adjuvant chemical is selected from one or more of;
- A) poly-ornithine, for example of molecular weight from 5 to 150kDa;
 - 35 B) vitamins or vitamin derivatives such as vitamin E TPGS (d-alpha tocophenyl polyethylene glycol 1000 succinate),

- C) cationic pluronics which are block copolymers or surfactants which are positively charged, in particular with NH_2^+ groups
- D) complexing agents which form complexes with fatty acids such as deoxycholic acid, or
- 5 E) cyclodextrins and their derivatives such as dimethyl β cyclodextrin.
5. A composition according to any one of the preceding claims wherein the carrier comprises a particle.
- 10 6. A composition according to claim 5 wherein the particle is a microsphere or liposome.
7. A composition according to claim 6 which comprises a
- 15 microsphere.
8. A composition according to claim 7 wherein the microsphere is prepared using a high molecular weight polymer.
- 20 9. A composition according to claim 8 wherein the polymer has a molecular weight of 100kDa or more.
10. A composition according to any one of claims 7 to 9 wherein the microsphere comprises poly-(L-lactide).
- 25 11. A composition according to any one of the preceding claims wherein the ratio of the chemical (ii) to the carrier is from 99:1 to 9:1 w/w.
- 30 12. A composition according to any one of the preceding claims which is adapted for administration to a mucosal surface or is suitable for parenteral administration.
- 35 13. A composition according to claim 2 which further comprises a further adjuvant.

14. A method of producing a prophylactic or therapeutic vaccine, which method comprises encapsulating a polypeptide which is capable of producing a protective immune response in a first polymeric material which has a high molecular weight, in
5 the presence of a second polymeric material which increases the biological effect of the composition.
15. A method of protecting a mammal against infection, which method comprises administration of a composition according to
10 any one of claims 1 to 13 to a mammal.
16. A method according to claim 15 wherein the composition is applied to a mucosal surface.
- 15 17. A method according to claim 16 wherein the mucosal surface comprises an intranasal surface.
18. A microsphere comprising an S-layer protein.
- 20 19. A microsphere according to claim 18 wherein said S-layer protein is coated on the surface of the microsphere.
20. A microsphere according to claim 18 or claim 19 which further comprises an agent that is capable of generating a
25 protective immune response in an animal to which it is administered.
21. A microsphere according to claim 20 wherein one or more of said agents are linked to the S-layer protein.
30
22. A pharmaceutical composition comprising a microsphere according to any one of claims 19 to 22.
23. A pharmaceutical composition according to claim 22 wherein
35 said composition is a vaccine, intended to produce a protective

immune response against a bacterium, and said S-layer protein is derived from said bacterium.

24. The use of a chemical selected from

- 5 A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,
- E) a complexing agent,
- 10 F) cetrinides;
- G) an S-layer protein; or
- H) Methyl-glucamine

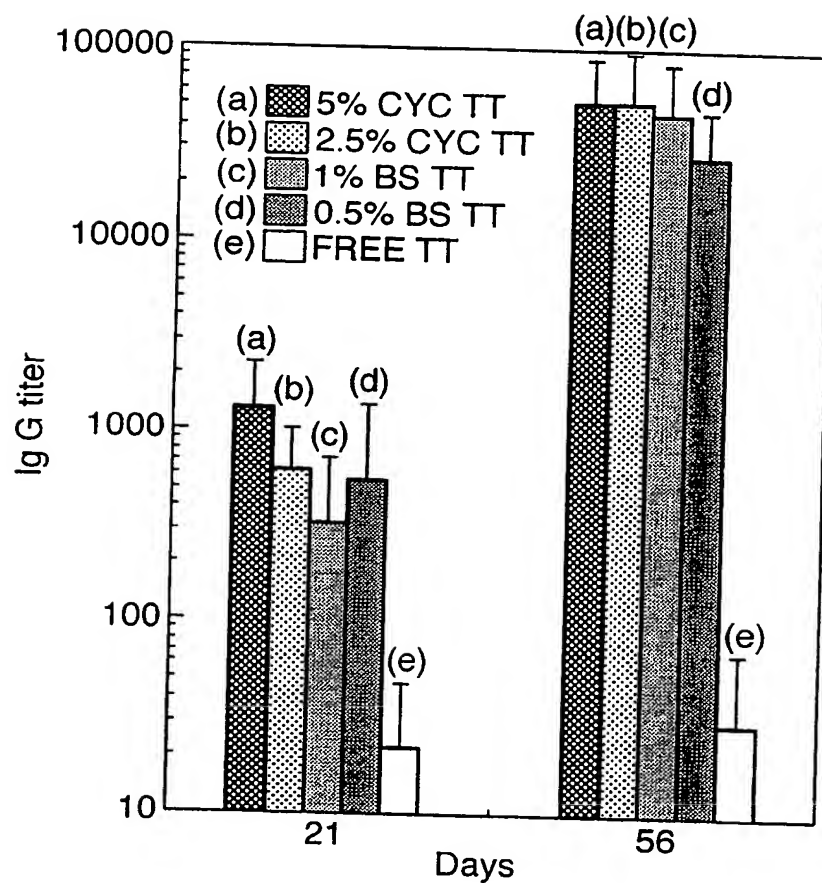
as an immunostimulant.

15 25. The use of an adjuvant chemical selected from

- A) a polyamino acid,
- B) a vitamin or vitamin derivative,
- C) cationic pluronics,
- D) a clathrate,
- 20 E) a complexing agent,
- F) cetrinides;
- G) an S-layer protein; or
- H) Methyl-glucamine

as an immunostimulant in the production of a vaccine for use in
25 prophylactic or therapeutic treatment.

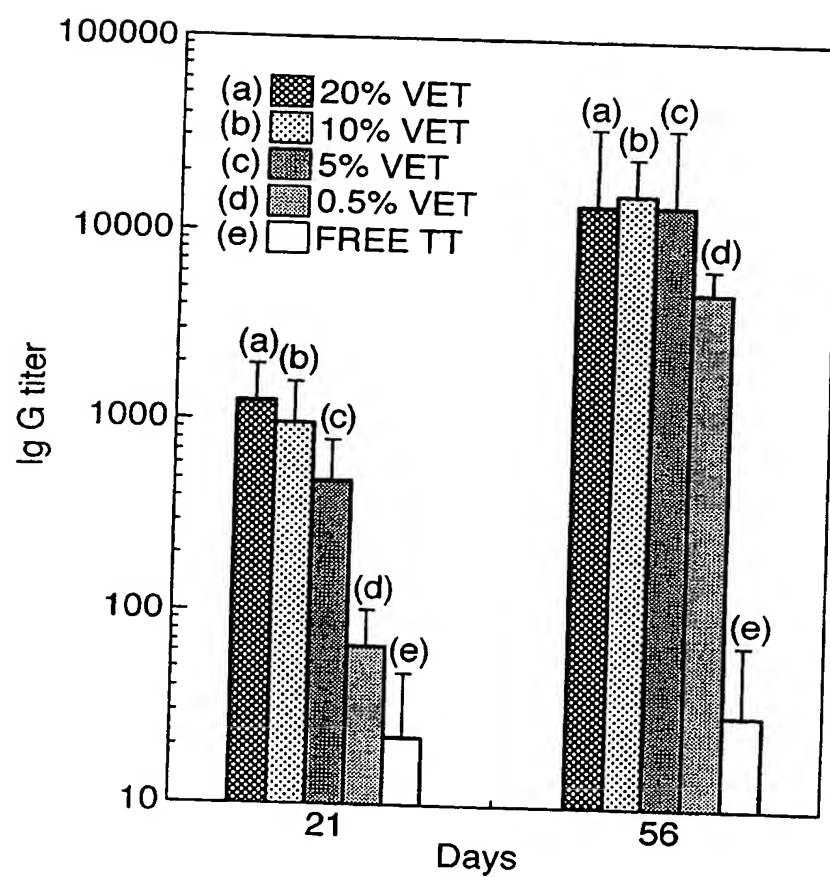
Fig.1.



Mice dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid

BS = Glyco deoxy cholic acid
CYC = Dimethyl (cyclodextrin)

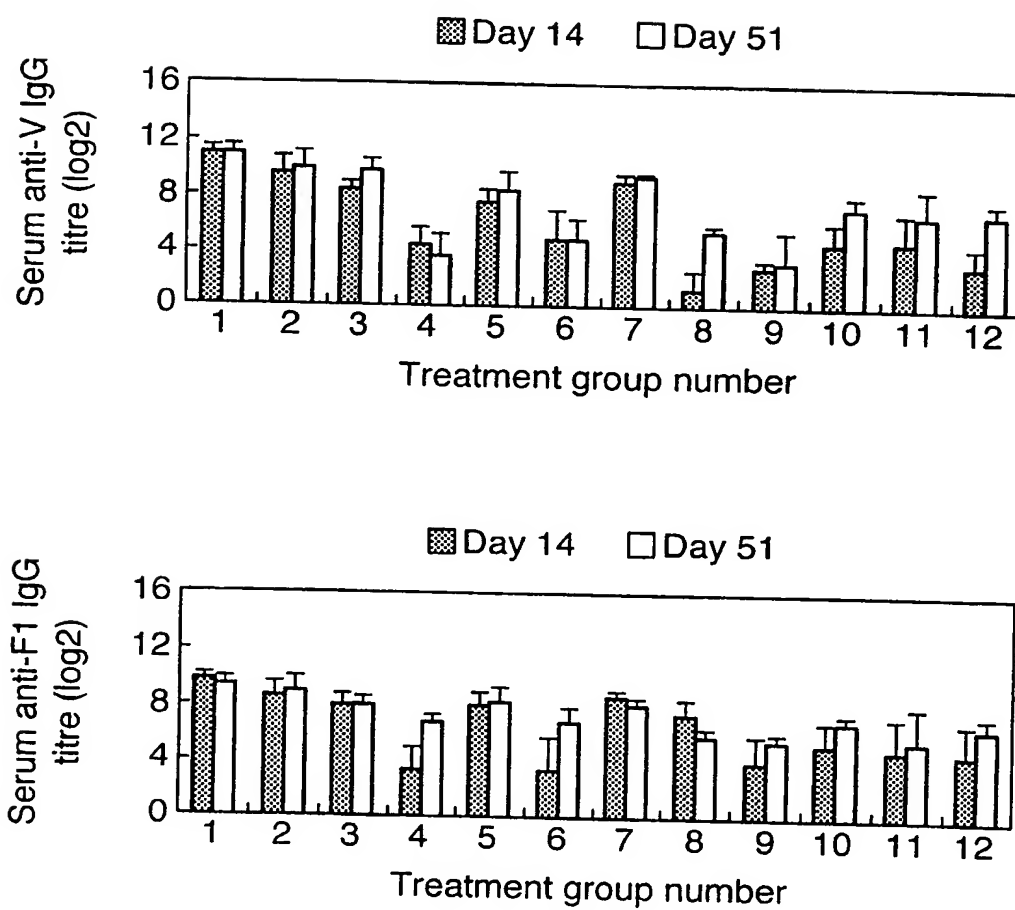
Fig.2.



Mice dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid

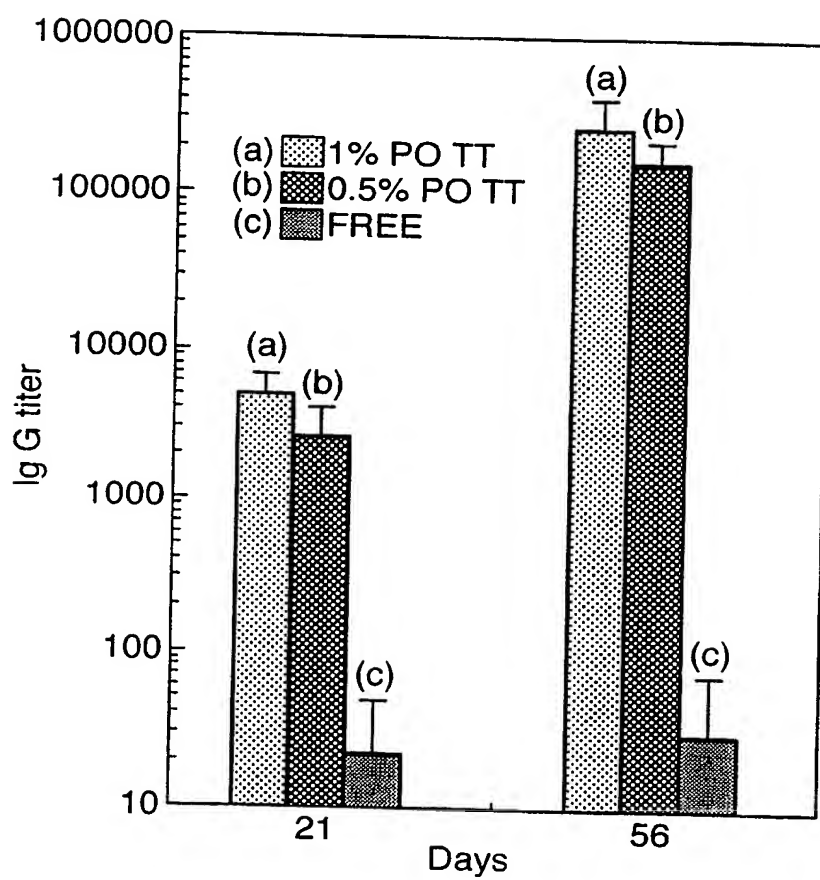
3/8

Fig.3.



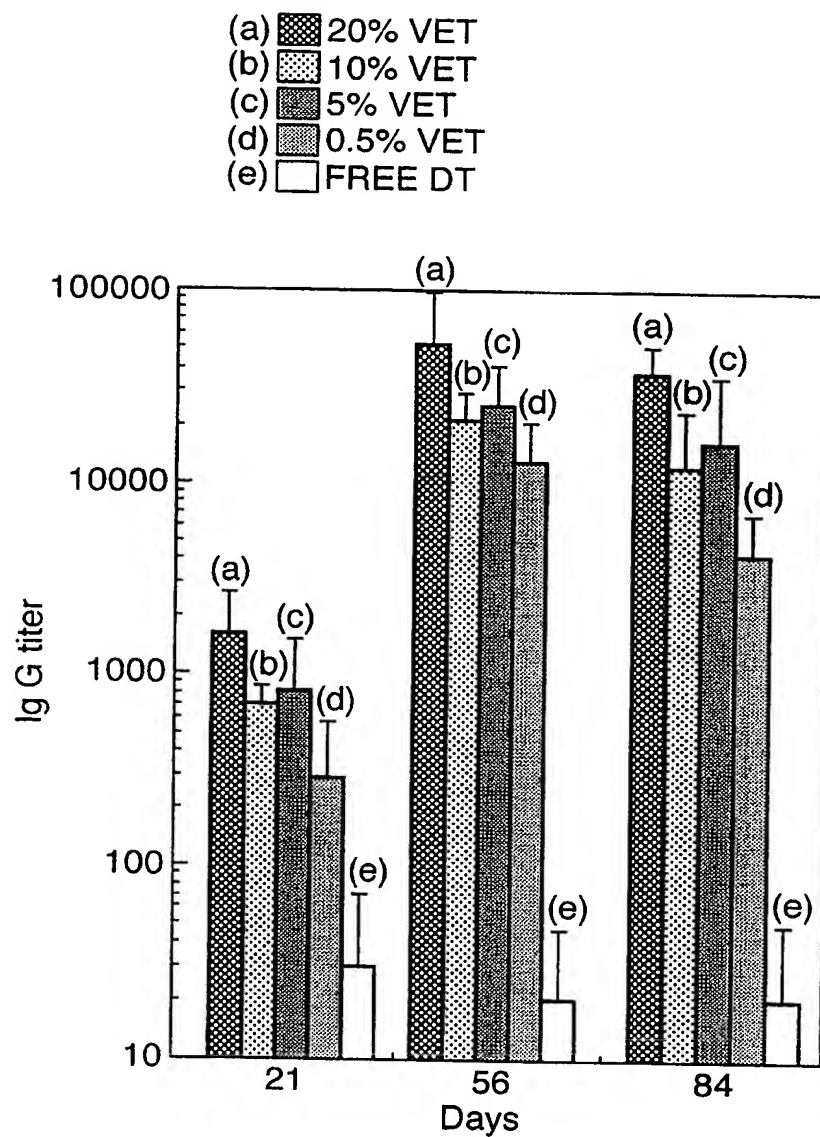
4/8

Fig.4.



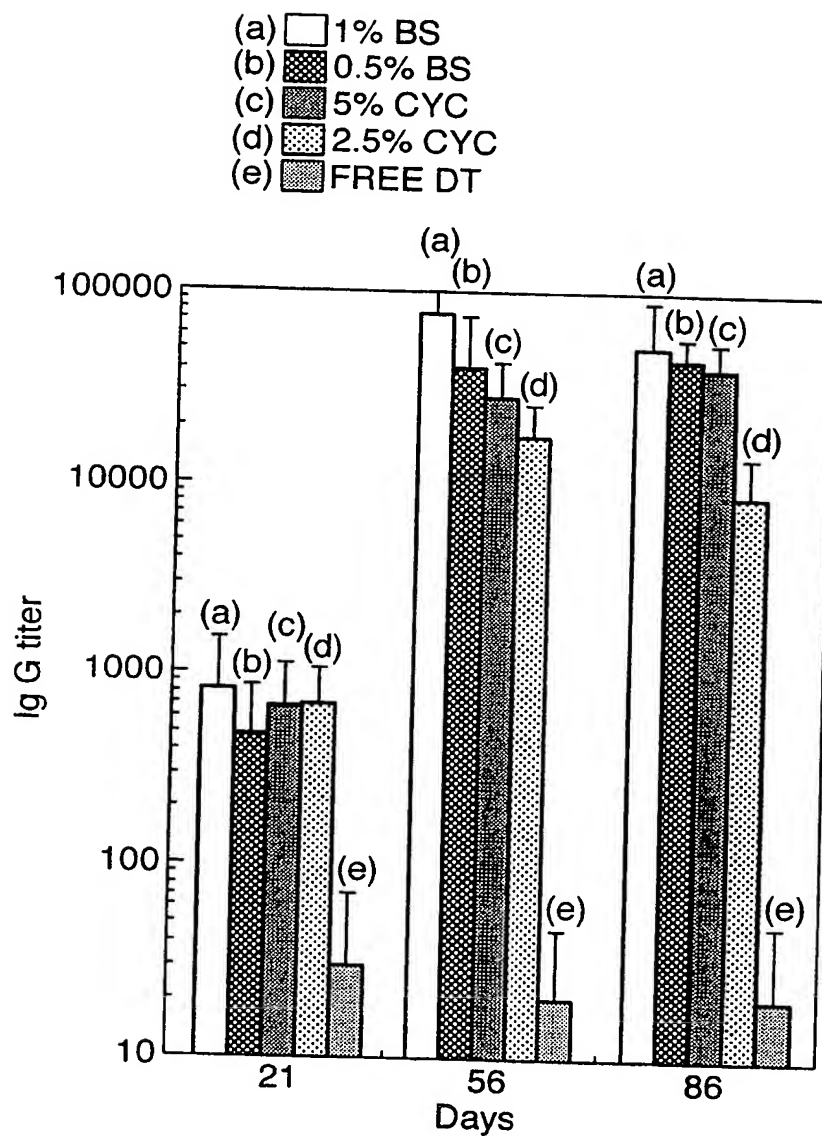
Mice dosed on day 1 with 5 LF toxoid and on day 49 with 2.5 LF toxoid

Fig.5.



Mice dosed on day 1 with 5 LF toxoid and on
day 49 with 2.5 LF toxoid

Fig.6.



Mice dosed on day 1 with 5 LF toxoid and on
day 49 with 2.5 LF toxoid

BS = Glyco deoxy cholic acid
CYC = Dimethyl (cyclodextrin)

SUBSTITUTE SHEET (RULE 26)

Fig.7.

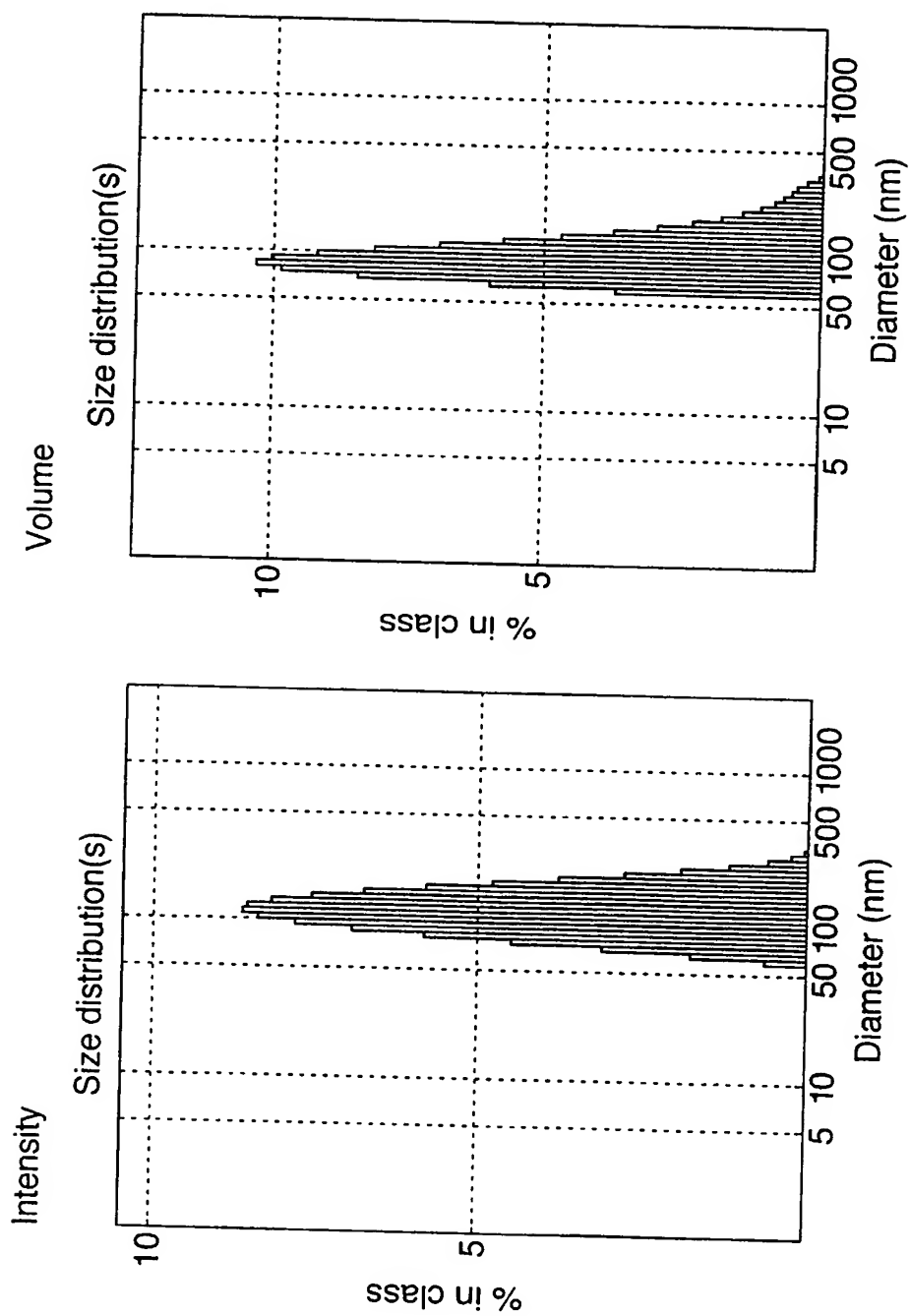
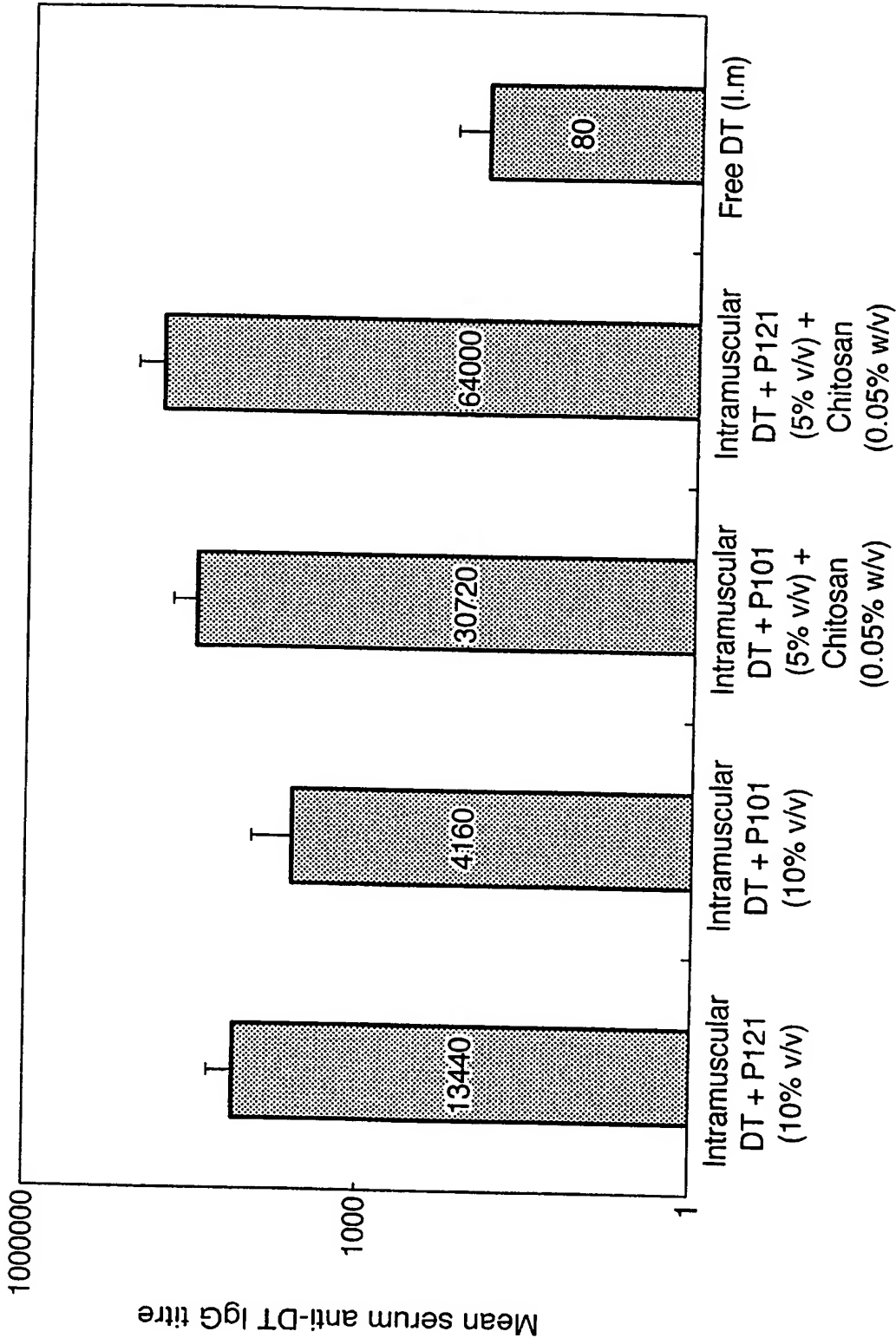


Fig.8.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 September 2000 (28.09.2000)

PCT

(10) International Publication Number
WO 00/56361 A3

(51) International Patent Classification⁷: A61K 39/39,
39/02, 9/16, 9/51, A61P 31/04

(74) Agent: BOWDERY, A., O.; D/IPR, Formalities Section,
Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

(21) International Application Number: PCT/GB00/01104

(22) International Filing Date: 23 March 2000 (23.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9906694.6 24 March 1999 (24.03.1999) GB
9906696.1 24 March 1999 (24.03.1999) GB

(71) Applicant (for all designated States except US): THE
SECRETARY OF STATE FOR DEFENCE [GB/GB];
Defence Evaluation and Research Agency, Ively Road,
Farnborough, Hampshire GU14 0LX (GB).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,
IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV,
MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO,
RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG,
US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): ALPAR, Hazire,
Oya [GB/GB]; Aston University, Aston Triangle, Birm-
ingham B4 7ET (GB). SOMAVARAPU, Satyanarayana
[IN/GB]; Aston University, Aston Triangle, Birmingham
B4 7ET (GB). WILLIAMSON, Ethel, Diane [GB/GB];
CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).
BAILLIE, Leslie, William, James [GB/GB]; CBD Porton
Down, Salisbury, Wiltshire SP4 0JQ (GB).

Published:

— With international search report.

(88) Date of publication of the international search report:
1 March 2001

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: VACCINE COMPOSITION

(57) Abstract: A pharmaceutical composition comprising: (i) a biologically active agent; (ii) an adjuvant chemical which increases the effect of the biologically active agent, said chemical selected from one or more of: A) a polyamino acid, B) a vitamin or vitamin derivative, C) cationic pluronics, D) a clathrate, E) a complexing agent, F) cetrimides, G) an S-layer protein, or H) methyl-glucamine; (iii) a pharmaceutically acceptable carrier or diluent, provided that when the chemical (ii) above is selected from D) or E), the biologically active agent is an agent which is capable of generating a protective immune response in an animal to which it is administered. The composition, which may be in the form of a solution or particles such as microspheres or liposomes, is particularly useful for mucosal administration of vaccines especially by the intra-nasal route or by parenteral routes.

WO 00/56361 A3

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 00/01104		
A. CLASSIFICATION OF SUBJECT MATTER IPC 7 A61K39/39 A61K39/02 A61K9/16 A61K9/51 A61P31/04		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 1 290 141 A (WELLCOME FOUNDATION) 20 September 1972 (1972-09-20) the whole document	1-5, 12, 15, 24, 25
X	FR 2 306 684 A (LABORATOIRES CRINEX) 5 November 1976 (1976-11-05) the whole document	1-5, 12, 15, 24, 25
X	US 5 650 155 A (LAMMERT C. ET AL.) 22 July 1997 (1997-07-22) the whole document	1-5, 12, 15, 24, 25
X	US 5 562 910 A (DAYNES R.A. ET AL.) 8 October 1996 (1996-10-08) the whole document	1-5, 12, 15, 24, 25
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center; font-weight: bold;">18 October 2000</div>		Date of mailing of the international search report <div style="text-align: center; font-weight: bold;">26/10/2000</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center; font-weight: bold;">Moreau, J</div>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01104

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 20070 A (SECRETECH) 15 September 1994 (1994-09-15) the whole document	1-5, 12, 15, 24, 25
X	EP 0 283 085 A (AKZO) 21 September 1988 (1988-09-21) the whole document	1-5, 12, 15, 24, 25
X	DE 196 03 649 A (LUBITZ W. ET AL.) 7 August 1997 (1997-08-07) the whole document	1-5, 12, 15, 24, 25
A	JAHN-SCHMID B ET AL: "Immunoreactivity of allergen (Bet v 1) conjugated to crystalline bacterial cell surface layers (S-layers)" IMMUNOTECHNOLOGY, NL, ELSEVIER SCIENCE PUBLISHERS BV, vol. 2, no. 2, 1 June 1996 (1996-06-01), pages 103-113, XP004052675 ISSN: 1380-2933 the whole document	1-25
A	EYLES J E ET AL: "Intra nasal administration of poly-lactic acid microsphere co-encapsulated Yersinia pestis subunits confers protection from pneumonic plague in the mouse" VACCINE, GB, BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 7, 1 April 1998 (1998-04-01), pages 698-707, XP004112258 ISSN: 0264-410X the whole document	1-25
A	MCBRIDE B W ET AL: "Protective efficacy of a recombinant protective antigen against Bacillus anthracis challenge and assessment of immunological markers" VACCINE, GB, BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 8, 1 May 1998 (1998-05-01), pages 810-817, XP004118489 ISSN: 0264-410X the whole document	1-25

	-/--	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01104

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p> GRIFFIN K F ET AL: "Immune responses to V antigen of Yersinia pestis co-encapsulated with IFN-gamma: effect of dose and formulation" VACCINE, GB, BUTTERWORTH SCIENTIFIC. GUILDFORD, vol. 16, no. 5, 1 March 1998 (1998-03-01), pages 517-521, XP004106965 ISSN: 0264-410X the whole document ----- </p>	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/01104

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 1290141 A	20-09-1972	NONE	
FR 2306684 A	05-11-1976	NONE	
US 5650155 A	22-07-1997	US 5667784 A AT 115862 T AU 633043 B AU 4897590 A CA 2008856 A CN 1044594 A,B DE 69015222 D DE 69015222 T DK 382271 T EP 0382271 A ES 2068989 T GR 3015438 T HU 56285 A,B JP 2250835 A JP 2892739 B KR 162646 B NZ 232354 A ZA 9000512 A	16-09-1997 15-01-1995 21-01-1993 09-08-1990 04-08-1990 15-08-1990 02-02-1995 04-05-1995 01-05-1995 16-08-1990 01-05-1995 30-06-1995 28-08-1991 08-10-1990 17-05-1999 01-12-1998 25-09-1991 28-11-1990
US 5562910 A	08-10-1996	US 5837269 A AU 679215 B AU 6234894 A CA 2153794 A CZ 9501975 A EP 0686042 A FI 953608 A HU 72404 A JP 8508718 T NO 953049 A NZ 262597 A PL 310112 A SK 97395 A WO 9417823 A US 5518725 A US 5824313 A US 5753237 A US 5919465 A AT 192651 T AU 652130 B AU 6501990 A AU 667018 B AU 7572794 A CA 2066716 A DE 69033541 D EP 0494224 A JP 2505313 B JP 5502856 T US 5540919 A WO 9104030 A US 5827841 A	17-11-1998 26-06-1997 29-08-1994 18-08-1994 13-12-1995 13-12-1995 19-09-1995 29-04-1996 17-09-1996 03-10-1995 24-10-1997 27-11-1995 08-05-1996 18-08-1994 21-05-1996 20-10-1998 19-05-1998 06-07-1999 15-05-2000 18-08-1994 18-04-1991 29-02-1996 08-12-1994 26-03-1991 15-06-2000 15-07-1992 05-06-1996 20-05-1993 30-07-1996 04-04-1991 27-10-1998
WO 9420070 A	15-09-1994	AU 692440 B AU 6361694 A BR 9405996 A	11-06-1998 26-09-1994 19-12-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/01104

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9420070 A		CA 2158040 A CN 1120310 A EP 0688205 A JP 8508247 T	15-09-1994 10-04-1996 27-12-1995 03-09-1996
EP 283085 A	21-09-1988	DE 3875762 A DE 3875762 T DK 141488 A ES 2052685 T GR 3007039 T HU 47224 A,B JP 2562827 B JP 63253032 A US 5026543 A US 5565209 A ZA 8801694 A	17-12-1992 13-05-1993 18-09-1988 16-07-1994 30-07-1993 28-02-1989 11-12-1996 20-10-1988 25-06-1991 15-10-1996 06-09-1988
DE 19603649 A	07-08-1997	AU 713999 B AU 1720397 A CA 2245584 A CN 1213402 A WO 9728263 A EP 0882129 A JP 2000503850 T NZ 331300 A	16-12-1999 22-08-1997 07-08-1997 07-04-1999 07-08-1997 09-12-1998 04-04-2000 29-06-1999